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Full Length Research Paper

In vitro antioxidant activity of acetone extracts from Chinese herb *agrimony* leaves

Zhe-xiong Jin and Bao-Qing Wang*

College of Pharmacy, Harbin University of commerce, Harbin, Heilongjiang Province, 150076, P R China.

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The purpose of this study was to evaluate antioxidant activities of the ethyl acetate soluble fraction (ESF) and butanol soluble fraction (BSF) of agrimony acetone extract. The ESF and BSF were investigated for their antioxidant activities by means of the 2, 2-diphenhyl-1-picrylhydrazyl (DPPH), 2, 2-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS), β-carotene-linoleate and hydroxyl radical assay. The IC50 values of ESF were 8.76, 7.28, 13.56 and 1.76 µg/ml, respectively in four assays. Similarly, BSF IC50 values were 9.77, 7.96, 8.61 and 2.57 µg/ml. The present study showed that both ESF and BSF have more effective antioxidant than butylated hydroxytoluene (BHT). It was concluded that agrimony might be a potential source of antioxidants.

Key words: Antioxidant activity, free radicals scavenging activity, agrimony.

INTRODUCTION

Antioxidants not only are commonly used as food additives to provide protection against oxidative degradation of foods, but also are used as a kind of traditional medicial active constituent to prevent some diseases. New developments in bio-medical science emphasis the involvement of free radicals in many diseases. There is increasing evidence to suggest that many degenerative diseases such as brain dysfunction, cancer, heart disease and immune system decline could be the result of cellular damage caused by free radicals and that antioxidants may play an important role in disease prevention (Baoqing , 2010). Recently, there has been intense attention in screening various plant extracts for natural antioxidants because of their good antioxidant properties and fewer side effects.

Medicinal plants are the main sources of natural antioxidants. Chinese herb agrimony called xianhecao and belonging to *Rosaceae* Family in China, is one of traditional Chinese medicine plant consumed in the eastnorth region of China (Zhe and Baoqing, 2010). This medical plant is traditionally used to suppress diarrhoea, reduce gastric ulcers, relieve fatigue, improve astringent effect and so on. It possesses many useful pharmacological activities including antiviral, anticancer, lowering blood sugar and hepatoprotective activities. These activities are likely related to their antioxidant properties of agrimony.

At present , there are two major types of antioxidants, and the first category is a natural antioxidants such as vitamin C, vitamin E, and relatively complex extracts from a number of plant species (Chun et al., 2010). The second category is mainly to synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ), but their use has been questioned because of their suspected toxic properties (Pan et al., 2008). The side effects of some synthetic antioxidants such as BHT and BHA have already been documented. For example, these substances can exhibit carcinogenic effects in living organisms (Wei et al., 2008). Therefore, China governmental authorities are concerned about the finding of natural antioxidant. Numerous researches are being carried out in order to obtain antioxidants in traditional Chinese herbs. The antioxidant capacity can be assessed by a number of chemical evaluation protocols. The commonly methods are DPPH and ATBS assays. DPPH method is one of the oldest and most frequently used methods for total antioxidant capacity of food and biological extracts. It is based on the ability of an antioxidant to give hydrogen radical to synthetic

*Corresponding author. E-mail: baoqingwang2002@yahoo.com.cn.

long-lived nitrogen radical compound DPPH (Enshyh et al., 2010). ATBS assay is mainly based on the following principle, the ferryl myoglobin radical can oxidize ABTS to generate a radical cation that is green in color and can be measured, Antioxidants suppress this reaction by electron donation radical scavenging and inhibit the formation of the colored ABTS radical.

Though a large number of plants worldwide show strong antioxidant activities, the antioxidant properties of Agrimony acetone extracts have not been elucidated before. The aims of the present work were to evaluate the activity of Agrimony acetone extracts using different methods. Several assays have been used to estimateantioxidant capacities in Agrimony acetone extracts including DPPH, ATBS, Hydroxyl radical and β-carotene-linoleate assays. Also, another purpose of this study was to compare antioxidative capabilities of agrimony acetone extracts with standard antioxidants of BHT. This work will provide a scientific foundation for further use of agrimony resources.

MATERIALS AND METHODS

Plant material

Agrimony was collected in may from the cap mountain of Harbin District, China, and was authenticated by Prof Jin zhe-xiong from College of Pharmacy, Harbin University of Commerce. Voucher specimens were deposited in the herbarium of this Laboratory. Leaves were air dried and then pulverised into a homogeneous size by a disintegrator and then sieved (30 to 40 mesh).

Preparation of extract

The acetone extract of sample was obtained by soaking 5 g of dried powdered samples in 175 mL of acetone for 15 min with microwave-assisted extract method. Then, the acetone extract was redissolved. The solution was partitioned successively in a separatory funnel with the ethyl acetate and n butanol to the corresponding soluble fractions. BSF is the butanol extract layer of agrimony acetone extract and ESF is the ethyl acetate extract layer of agrimony acetone extract. Different extract fractions were evaporated to dryness and stored in the dark at 4℃ until the experiment was carried out. BHT is synthetic antioxidant butylated hydroxytoluene.

DPPH radical scavenging activity

The DPPH radical scavenging activity of the acetone extracts from agrimony, as well as positive control BHT was measured using the method of Brand-Williams (Brand et al., 1995), with slightly modified as follows: a 2.0 mL of methanol solution of DPPH (0.2 mmol/L) was mixed with equivalent aliquot of different samples and after standing in dark for 30 min, and absorbance at 517 nm was measured against methanol. Controls containing methanol instead of the sample and blank containing methanol instead of DPPH solution were also measured, respectively. Positive control BHT was used for comparative purpose. The inhibition of the DPPH radical by the samples was calculated according to the following formula:

DPPH scavenging activity (%) = [1-(Abs. of sample - Abs. of blank)/Abs. of control] ×100

$ABTS^+$ radical scavenging activity

The $ABTS^+$ radical scavenging activity of agrimony acetone extract was determined according to the improved assay of ReR (Re et al., 1999) with some modifications. $ABTS^+$ was dissolved in PBS (0.01 M, pH 7.4) to a 7 mM concentration. ABTS radical was produced by mixing the solution with 2.45 mM potassium persulfate and allowing the mixtures to stand in the dark at room temperature for 12 to 16 h. The $ABTS^+$ solution was diluted with PBS to an absorbance of 0.70 ± 0.05 at 734 nm and equilibrated at 30℃ for 30 min. A methanolic solution (0.2 ml) of different sample was mixed with 2.0 ml of diluted $ABTS^+$ solution. After 20 min reaction at room temperature, the absorbance at 734 nm was measured. The radical scavenging activity of the sample was expressed as:

$$\text{Inhibition\%} = [(A_{control}-A_{test})/A_{control}]\times 100\%,$$

Where $A_{control}$ is the absorbance of the control ($ABTS^+$ solution without test sample) and A_{test} is the absorbance of the test sample ($ABTS^+$ solution plus extracts). The amount of the extract in ug/ml at which the absorbance decreased to half its initial value was taken as IC50 value for the extract.

Antioxidant activity assay with the β-carotene-linoleate model system

The antioxidant activity was evaluated according to following Procedures. Generally, a 0.2 mg sample of β-carotene in 0.2 ml of chloroform, 20 mg of linoleic acid, and 200 mg of Tween-40 were mixed. Chloroform was removed at 40℃ under vacuum and the resulting mixture was diluted with 10 mL of water and mixed well. This emulsion was added 40 mL of oxygenated water. Four-milliliter aliquots of the emulsion were pipetted into different test tubes containing 0.2 ml of different concentrations of solvent fractions and subfractions. Controls containing 0.2 ml of solvent and 4 ml of the above emulsion were prepared. The tubes were placed at 50℃ in a water bath and the absorbance at 470 nm was taken at zero time (t = 0). The measurement of absorbance was continued at intervals of 30 min until the color of β-carotene disappeared in the control tubes (t = 120 min). A mixture was prepared as above without β-carotene as the blank. BHT was used for comparative purpose. All determinations were carried out in triplicate. The antioxidant activity (AA) of the samples was evaluated in terms of bleaching of the β-carotene, using the following formula:

$$AA(\%) = [1-(A_0 - A_t)/(A^0_0 - A_t^0)] \times 100$$

Where A_0 and A^0_0 are the absorbance values measured at zero time of the incubation for the test sample and the control, respectively, and A_t and A_t^0 are the absorbance values measured in the test sample and the control, respectively, after incubation for 120 min (Huang et al., 2007).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of BSF, ESF and BHT was estimated through the new method of Liang et al. (2006). HCl-NaAc buffer solution (pH = 4.95, 1.0 ml), KI solution (0.020 mol/L, 0.7

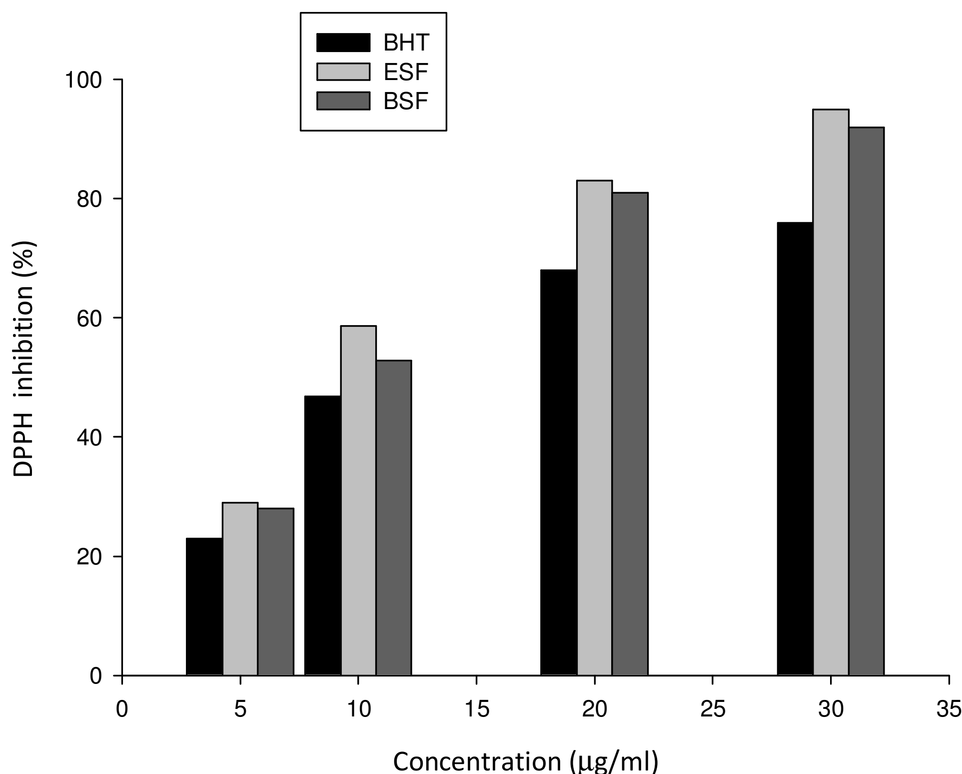

Figure 1. DPPH assay of Acetone extracts from agrimony.

ml), Fe(II) solution (4.00×10^{-5} mol/L, 0.1 mL) and H_2O_2 standard solution (6.48 umol/L, 0.4 mL) were piped in a 10 ml graduated tube, then rhodamine B (RhB,1.50×10^{-4} mol/L, 1.4 ml) was mixed. The mixed solution was diluted to 5 mL with water and mixed thoroughly. The resonance scattering (RS) spectra was obtained by using the synchronous scanning technique in spectro-fluorophotometer. The A1 which presents the RS intensity for the system containing H_2O_2 and scavenger, A presents the RS intensity for the system containing H_2O_2 and A2 the RS intensity in the absence of H_2O_2 were measured at 420 nm. The scavenging rate could be calculated as:

$$(\%) = [(A-A1)/(A-A2)] \times 100\ (\%)$$

The IC50 defined as the concentration of sample at which 50% of hydroxyl radical was scavenged and calculated for each sample.

Statistical analysis

Experimental analyses were performed in triplicate. Data were recorded as mean ± standard deviation and analyzed by SPSS. Statistical significance was considered at $p< 0.05$.

RESULTS AND DISCUSSION

DPPH assay

Antioxidant properties, especially radical scavenging activity is very important, due to the deleterious role of free radicals in plants and in biological systems. Diverse methods are currently used to assess the antioxidant activity of agrimony acetone extracts. The DPPH radical is a stable organic free radical with a maximum absorption at 517 nm, and it has been extensively used for screening antioxidants from fruit and vegetable juices or natural extracts (Pendry et al., 2005). The DPPH radical scavenging activity (%) of ESF and BSF of agrimony acetone extract, compared to BHT are shown in Figure 1. The concentrations providing 50% inhibition (IC50) values of ESF, BSF and BHT were 8.76, 9.77 and 12.09 µg/ml respectively. All IC50 results are summarized in Table 1. Obviously, both ESF and BSF of agrimony acetone extract showed excellent activities compared with BHT, which has been confirmed to possess powerful antioxidative activity. It seemed that ESF were higher effective in scavenging activities than BSF. In other word, the scavenging effect on the DPPH radical decreased in that order: ESF > BSF> BHT.

ABTS assay

It is known that ABTS is an excellent substrate for peroxidases frequently used to study antioxidant

Table 1. IC50 (μg/ml) Value in four assays.

Sample	DDPH assay	ABTS assay	βcarotene assay	Hydroxyl radical
ESF	8.76	7.28	13.56	1.76
BSF	9.77	7.96	8.61	2.57
BHT	12.09	9.88	29.25	4.53

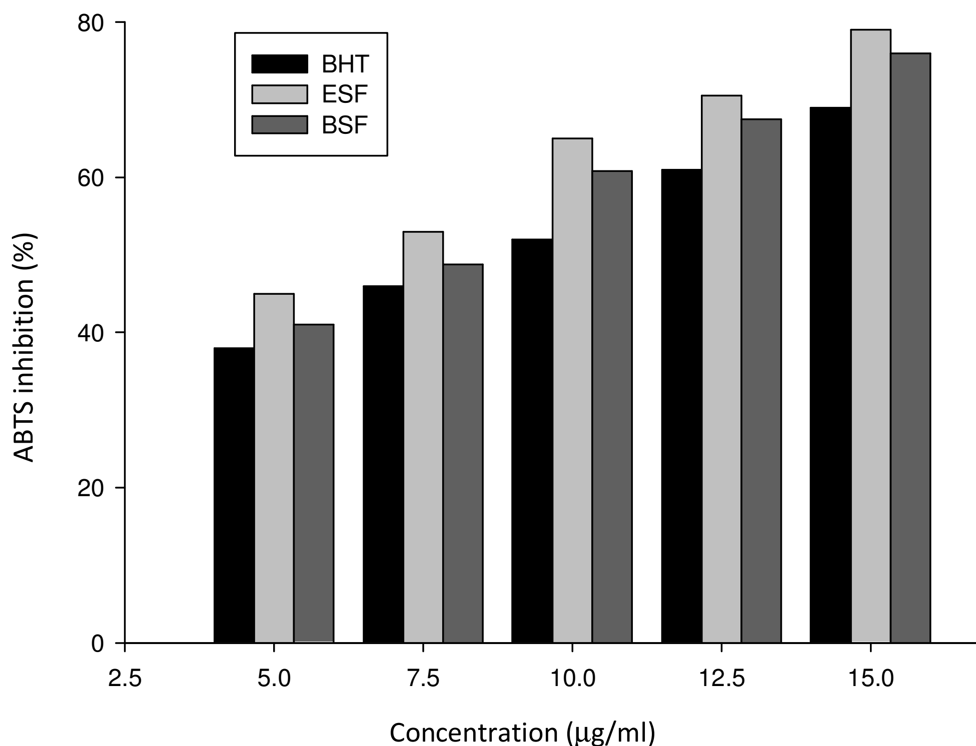

Figure 2. ABTS assay of Acetone extracts from agrimony.

properties of natural compounds (Reszka and Britigan, 2007). In order to evaluate the antioxidant potency through free radical scavenging by agrimony acetone extracts, the change of optical density of ABTS radicals was monitored. And BHT was used as standards. Figure 2 depicts the ABTS radical scavenging activity of ESF and BSF as well as BHT. The activity of ESF in scavenging the ABTS radical was good, with an IC50 value of 7.28 μg/ml, which was lower than that of BSF (IC50 value of 7.96 μg/ml). BSF showed a higher potency than BHT (IC50 value of 9.88 μg/ml). Thus, the ABTS radical scavenging activity decreased in the following order: ESF>BSF>BHT. This result is similar with DPPH assay. Thus the radical-scavenging capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Our result demonstrated that both BSF and ESF have significant scavenging activity on DPPH and ABTS radicals.

β-carotene-linoleate assay

In this assay system, β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange colour, a change which can be monitored spectrophotometrically (Jayaprakasha et al., 2001). The presence of samples can hinder the extent of b-carotene-bleaching by scavenging the linoleate-free radical and other free radicals formed in the system. Figure 3 shows the antioxidant activities of ESF, BSF and BHT determined by the β-carotene-linoleate model system. The result showed that ESF also possess better antioxidant activity than BHT, but it was not as good as BSF. The scavenging effects of ESF, BSF and BHT decrease in the following orders: BSF (IC50 value of 8.61 μg/ml) > ESF (IC50 value of 13.56 μg/ml) > BHT (IC50 value of 29.25 μg/ml), which is different from the result of the DPPH

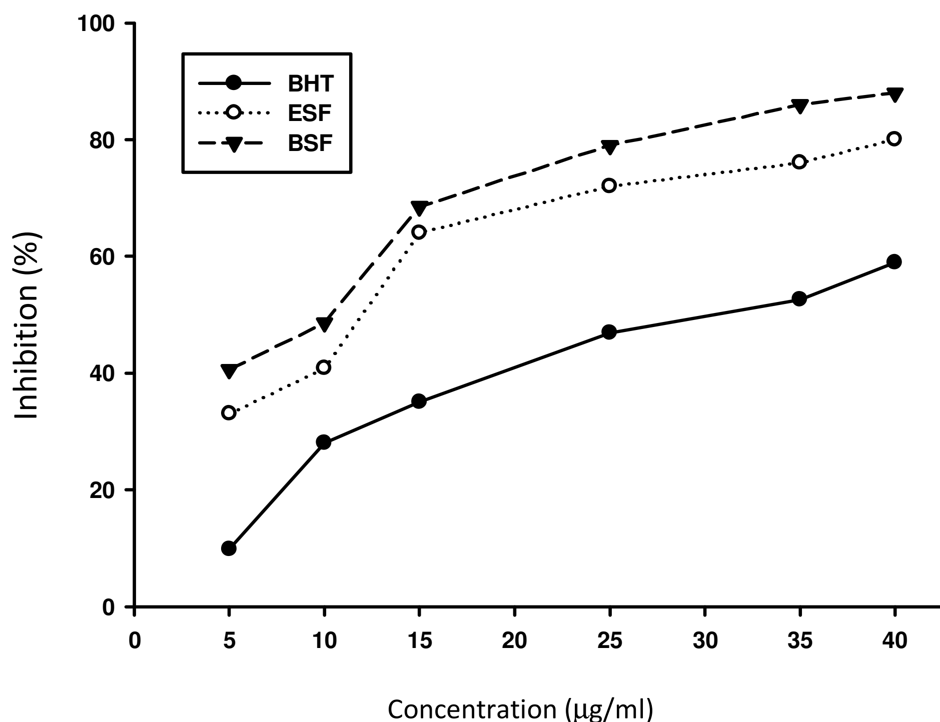

Figure 3. β-carotene-linoleate assay of Acetone extracts from agrimony.

radical-scavenging activity assay and the ABTS radical-scavenging activity assay. This reason may be BSF contain some special chemical constituents which can react with linoleic acid.

Hydroxyl radical assay

Hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism among the reactive oxygen species, which could be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron and cause the aging of human body and some diseases (Siddhuraju and Becker, 2007). Hydroxyl radical formation can occur in several ways; by far the most important mechanism *in vitro* is the Fenton reaction where a transition metal is involved as a prooxidant in the catalyzed decomposition of superoxide and hydrogen peroxide (Stohs and Bagchi, 1995). In this study, hydroxyl radical scavenging activity of samples was measured by a new resonance scattering spectral method from Fenton reaction.

Figure 4 indicates that the ESF and BSF exhibited a quite strong inhibition of hydroxyl radical at a pretty low concentration compared to BHT, and the scavenging percentage of ESF was better than BSF. The IC50 of ESF was only 1.76 μg/ml, and the IC50 of BSF was 2.57 μg/ml whereas BHT reached 4.53 μg/ml. So the scavenging ability was in this order: ESF > BSF > BHT. It is, thus, anticipated that ESF and BSF would scavenge hydroxyl radicals at different stages.

Conclusion

In order to evaluate the antioxidant activity of a natural product, it is crucial to implement more than one antioxidant methods, taking into consideration the various oxidation aspects in the systems under scrutiny (Mohammad and Ali, 2010). In this study, DPPH radical scavenging, ABTS radical scavenging, β-carotene-linoleate assay and Hydroxyl radical assay were applied to evaluate the antioxidant activity of ESF and BSF from agrimongy acetone extract *in vitro* assays. BSF and ESF show better antioxidant and free radical scavenging activities than BHT, which may be due to the presence of tannin and polyphenol in agrimony acetone extract. Further studies regarding the isolation and characterization of active antioxidative constituents in agrimony acetone extract is currently under progress in our research group. In light of these valuable results, leaves of agrimony considered as waste materials have a good commercial potential to be utilized as promising natural antioxidants in the food, pharmaceutical or cosmetic industries, not only for the low cost but also for

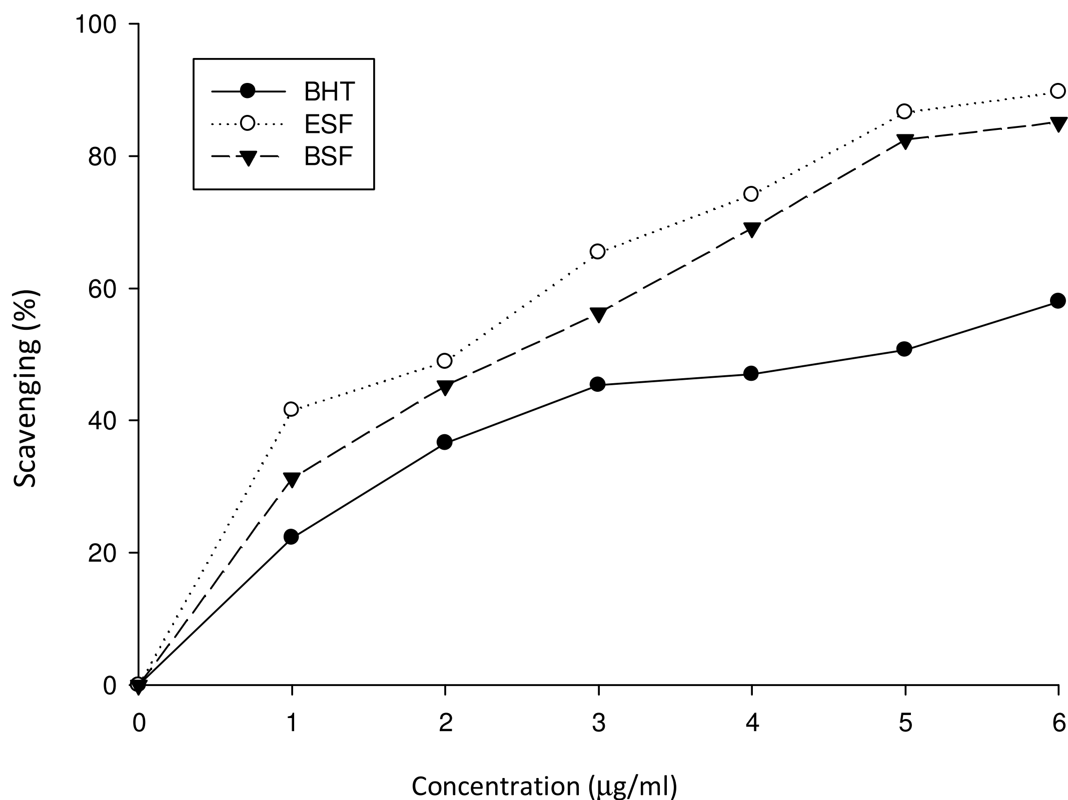

Figure 4. Hydroxyl radical assay of Acetone extracts from agrimony.

the large amounts available.

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